Jordan Journal of Pharmaceutical Sciences, Volume 17, No. 1, 2024

Formulation and Evaluation of Herbal Emulsion-Based Gel Containing Combined Essential Oils from *Melaleuca alternifolia* and *Citrus hystrix*

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ABSTRACT

Aims: This study aimed to investigate the synergistic anti-inflammatory effects of combined essential oils from Melaleuca alternifolia and Citrus hystrix peel.

Methodology: An emulsion-based gel containing combined essential oils from Melaleuca alternifolia and Citrus hystrix peel was topically applied to the injected area of the carrageenan-induced hind paw edema mouse model. Diclofenac sodium was used as a positive control. The inhibition percentage of protein denaturation in all mice was assessed and calculated based on paw volume (Pv).

Results: The highest inhibition percentage, recorded at the third hour in the carrageenan-induced hind paw edema mouse model, was 44.76%, achieved by the mice treated with combined essential oils. This was followed by diclofenac-treated (37.14%), tea tree essential oil-treated (27.62%), and kaffir lime peel essential oil-treated mice (20.10%). A similar trend was observed after 24 hours of treatment, with the anti-inflammatory activity of combined essential oils-treated mice recorded at 46.74%, followed by diclofenac-treated (35.87%), tea tree essential oil-treated (24.97%) and kaffir lime peel essential oil-treated mice (11.97%).

Conclusion: These findings confirm the synergistic anti-inflammatory effects of the combined essential oils-incorporated emulsion-based gel on the carrageenan-induced hind paw edema mouse model.

Keywords: *Melaleuca alternifolia*, *Citrus hystrix*, essential oil, anti-inflammation, carrageenan-induced, edema.

INTRODUCTION

According to the World Health Organization (WHO), medicinal plants could become the best source to obtain a variety of medicine [1]. Approximately 80% of individuals from developed countries use traditional medicine, which contains compounds derived from medicinal plants [2]. Essential oils are aromatic oily liquids (also known as volatile oils) obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots) [3]. Of the estimated 3,000 known essential oils, some have been used for the treatment of various diseases while others have been utilized in food preservation or fragrance industries. Essential oils and their components are gaining increasing interest due to their ready availability, non-toxicity, and safety.

Melaleuca alternifolia (M. alternifolia) essential oil or tea tree oil (TTO) is a mixture of natural volatile constituents mainly extracted from the leaves of M. alternifolia, an evergreen shrub growing up to 6 m, predominantly found in Australia [4,5]. TTO is naturally present throughout the year in the leaf, but only found in the flower in June. TTO is recognized as a potential agent against microbial species and is now commonly used in pharmaceutical and cosmetic applications. TTO consists

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Received: 1/8/2023 Accepted: 21/12/2023.
DOI: https://doi.org/10.35516/jjps.v17i1.1570

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of many terpenes and their derivatives, of which terpinen-4-ol, α-pinene, linalool, and α-terpineol are considered the most important components having an antimicrobial effect [6, 7]. As revealed in prior studies, TTO exhibits a wide spectrum of antimicrobial activity against pathogenic microorganisms including Gram-positive and Gram-negative bacteria, yeasts, and fungi. Additionally, TTO has shown promise as a growth-inhibiting agent against multi-resistant microbes. Furthermore, TTO has been found effective in reducing inflammation, and is consequently widely incorporated as an active ingredient in numerous topical formulations for the treatment of various skin conditions such as wounds, acne, and contact dermatitis [8]. Given that high concentrations of TTO have been linked to irritation and side effects in humans, the combination of TTO with another essential oil such as Citrus limon leaves, Piper nigrum leaves [9], or Piper beetle leaves [10] is being researched. This can potentially lower the required dosage of TTO while also enhancing the therapeutic efficacy of blended essential oils.

Citrus hystrix (C. hystrix), or kaffir lime, is an evergreen shrub that grows up to 3 meters at a moderate rate. Its flowers are pollinated by insects and are self-fertile. Essential oil from the peel of C. hystrix, or kaffir lime peel essential oil (KPO), has been found to possess various significant biological activities and is touted for topical applications. Moreover, the essential oil derived from the peels and leaves is extensively used in the cosmetics and pharmaceutical industries, due to their significant pharmacological properties. These properties are primarily attributed to major phyto-constituents present in KPO, including β-pinene, α-pinene, D-limonene, and terpinen-4-ol [11, 12]. Additionally, KPO demonstrates inhibitory effects on human skin enzymes, including tyrosinase and hyaluronidase [13]. According to the Food and Agriculture Organization (FAO) statistics, roughly 60% of 998.7 thousand tons of citrus fruit waste is discarded per year in Vietnam [14]. Hence, using the waste peel from citrus fruits has gained considerable interest from researchers, due to its environmentally friendly nature, for developing C. hystrix-based healthcare products like soap [15] and mouthwash [16]. This study was primarily focused on investigating the synergistic anti-inflammatory efficacy of a gel incorporating a combination of tea tree and kaffir lime essential oils.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were procured from the Pharmaceutical Chemistry Laboratory and Applied Biochemistry Laboratory of the Applied Biochemistry Department in International University Ho Chi Minh City, Vietnam. Carrageenan, triethanolamine (TEA), bovine serum albumin (BSA), Tween 80, and Carbomer 940 were bought from Sigma-Aldrich (USA). Phosphate buffered saline (PBS) buffer was prepared by dissolving a PBS tablet from Merck (Germany) in 1 liter of deionized water. Glycerol was a product of Merck (Germany), and Diclofenac sodium 75 mg/3mL was a product of Voltaren (Novartis, Switzerland). All chemicals and reagents were stored according to stringent regulations and freshly prepared with distilled water at desired concentrations for experimental purposes.

Extraction of Essential Oils

Fresh M. alternifolia leaves and C. hystrix peels were collected from a farm in Southern Vietnam, then thoroughly washed with running water to eliminate potential contaminants. The plant materials were air-dried and cut into small pieces prior to the extraction of the essential oil. These fresh plant materials were subjected to microwave-assisted extraction using a NEOS Milestone appliance for 90 minutes under the power of 900 W (yields an extract temperature of 100-110°C) [17, 18]. The resulting volatile oils were stored in amber vials and refrigerated at 4°C until analysis.

In vitro Anti-inflammation Assay

The anti-inflammatory properties of KPO and TTO were evaluated using a modified anti-protein denaturation

of the bovine serum albumin assay [19]. This assay was prepared by mixing 0.5 mL of test solution, comprised of 0.45 mL of BSA (5% w/v aqueous solution), and 0.05 mL of each essential oil at varying concentrations. Diclofenac sodium was chosen as a positive control, while de-ionized water was used as a negative control. All solutions were adjusted to a pH of 6.3 using 1N hydrochloric acid (109057, Merck, Germany). The samples were incubated at 37°C for 30 minutes, after which the temperature was increased to 57°C for another 3 minutes. Subsequently, the reaction mixture was allowed to cool at room temperature before adding 2.5 mL of PBS buffer. The absorbance was recorded at 660 nm using a spectrophotometer (Therma Scientific, USA) and a BioTek Synergy HT microplate reader. The percentage of inhibition of protein denaturation was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Abs control solution-Abs test solution}}{\text{Abs control solution}}$$

The calibration curve was established using the regression equation y = bx + a. The anti-inflammatory activity of the essential oil was expressed as an IC50 value (μg/mL), which represented the concentration of essential oil causing 50% inhibition of protein denaturation. This was then compared with a sample containing the reference drug, diclofenac sodium 75 mg/mL.

Emulsion-based Gel Formulation

The emulsion-based gel, referred to as "emulgel," was formulated following the design protocol of Wulansari, Jufri, & Budianti, 2017 [20]. It was prepared in two phases using the basic ingredients as detailed in Table 1. The oily phase was prepared by dissolving Tween-80 in distilled water, followed by the addition of the essential oils (either KPO and TTO separately or in combination). Glycerol was added to this mixture and homogenized at 2,000 rpm for 15 minutes. In a separate container, Carbomer 940 was dispersed in distilled water before the addition of TEA to attain a pH in the range of 5.0-6.5. The preparation of the emulgel continued as the oily phase was combined with the aqueous phase under continuous agitation. This was completed by adding distilled water to make up 100 g of the mixture. The combination was homogenized using a homogenizer for 30 minutes until a smooth, clump-free gel with good integrity was obtained. The resultant gel formulations were transferred into sterile airtight containers, sealed, and stored in a cool area.

Table 1. Formulation of emulgel

	Ingredient	Amount (%)
Oily phase	Tween 80	30.00
	Glycerol	20.00
	Essential oil (individual or combined)	KPO: 3.501 g TTO: 2.717 g
Aqueous phase	Carbomer 940	1.00
	TEA	pH adjuster
	Distilled water	Qs. 100%

Evaluation of Emulgel Formulation

Physical Evaluation

Physical characteristics including color, odor and feeling [21] of application of prepared emulgel were noted.

Determination of pH

The pH values were determined using a digital pH meter [20]. This was done by dissolving 1 g of the emulgel sample in 100 mL of deionized water. Before the pH measurements, the pH meter was calibrated using standard

buffer solutions (pH 4, 7, and 10). Each preparation was measured in triplicate.

Homogeneity

The sample was subjected to visual observation by smearing it on microscope slides at three different temperatures (4, 25, and 37 °C) [22]. The presence of any clumps or disintegration would be considered a sign of heterogeneity [23, 24, 25].

Viscosity

The viscosity of the preparation was measured using a Brookfield viscometer Model RV-E with a spindle S64, at a rotation rate of 0.6 rpm at 4, 25, and 37°C [26].

Spreadability

Spreadability was determined using an apparatus [27] made up of a wooden block controlled by a pulley, based on the slip and drag characteristics of the gel. An excess of gel (about 2 g) was placed on a fixed slide. This gel sample was then sandwiched between this slide and another of the same dimensions attached to a hook. A 1-kg weight was placed on top of the two slides for 5 minutes to expel air and provide a uniform gel film between the slides. The excess gel was scrapped off from the edges. The top plate was then subjected to a pull of 80 g. Spreadability was calculated using the following formula:

$$\mathbf{S = M \times L / T}$$

Where S = Spreadability; M = Weight in the pan (tied to the upper slide); L = Length moved by the glass slide; and T = Time (in seconds) taken to separate the upper slide from the ground slide.

Stability

The stability of the sample was evaluated by observing changes in appearance, homogeneity, pH, spreadability, and viscosity at 25°C for a duration of 2 months.

Animal Preparation for Testing

Albino Wistar mice of either sex, weighing 27 ± 2 g, were procured from the Pasteur Institute (Ho Chi Minh, Vietnam) and allowed to acclimate in experimental conditions for a week. All animals were approved and maintained according to the Animal Experimental Handbook at the Cellular Reprogramming Laboratory, International University, Vietnam National University of Ho Chi Minh City (http://crl.bio.hcmiu.edu.vn/about-us/facilities/) [28] and in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition) [29]. Mice were kept in a controlled environment with a light and dark cycle of 12 hours, provided a standard diet and water ad libitum. Animals were fasted for 24 hours preceding the assay.

Skin Irritation Assay

The intact skin of the prepared mice was used to create an animal model. Back hairs were removed three days before the experiment. Animals were treated with the prepared emulgel daily for a week before the erythema and edema on the treated skin were examined [27, 30]. The Primary Dermal Irritation Index (PII) was calculated based on the number of erythema scores recorded for each period of time in order to classify the degree of irritation [31]:

$$\text{PII} = \frac{[(\Sigma \text{ erythema scores at } 1/24/48/72\text{h/day } 7 + \Sigma \text{ edema scores at } 1/24/48/72\text{h/day } 7)]}{5 \times \text{number of mice}}$$

Induction of Hind Paw Edema by Carrageenan and Assessment of Anti-inflammatory Activity on Mice Model

Mice were randomly divided into six groups of eight (n = 8). Group I was the physiological group (normal mice). Group II was treated with 5 mg/kg body weight of diclofenac (positive control). Group III was treated with the emulgel incorporating combined essential oils. Group IV was treated with the KPO-incorporated emulgel, while group V was treated with the TTO-loaded emulgel. Group VI was the negative control group. The induction of hind paw edema in mice was conducted by injecting 50 µL of 1% w/v carrageenan in saline solution into the plantar side of the right hind paw of the mice, one hour before each experiment. A test sample, consisting of 0.2 g of the emulgel or diclofenac, was topically applied onto the injected area of the hind paw and gently rubbed in using a gloved finger. Paw volume (Pv) was measured using a

Plethysmometer (Ugo Basile, Italy) at 0, 3, and 24 hours after administration. The percentage of inhibition of protein denaturation was calculated based on Pv using the following formula:

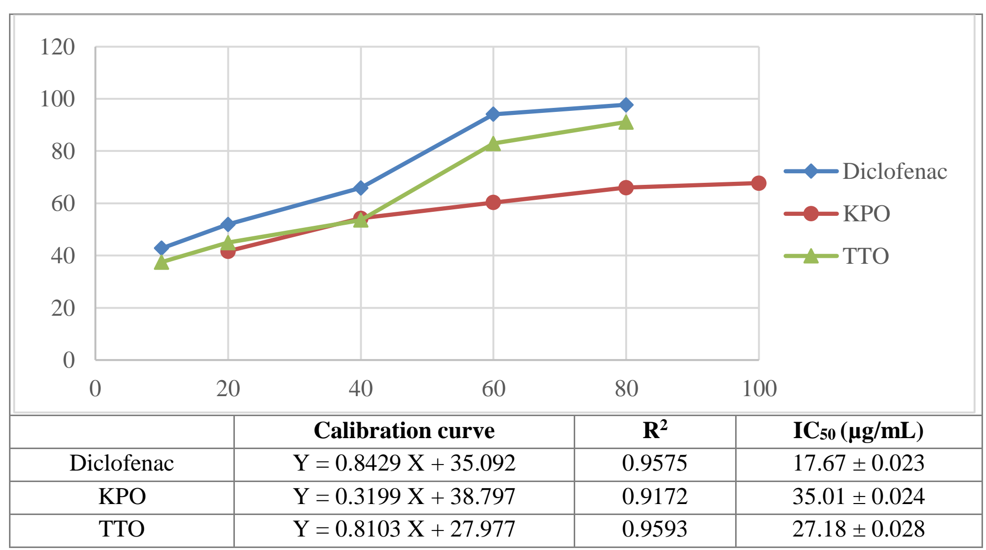
$$\% \, Inhibition = \frac{(Pv \, control - Pv \, test)}{Pv \, control} \times 100\%$$

Statistical Analysis

All experiments were conducted in triplicate. All values were reported as mean ± SD (standard deviation) and compared using an analysis of variance (ANOVA) for single-factor experiments. This was followed by Tukey's test to examine the significant difference between experimental data. Values with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In vitro Anti-inflammation



	Calibration curve	R^2	IC_{50} (µg/mL)
Diclofenac	Y = 0.8429 X + 35.092	0.9575	17.67 ± 0.023
KPO	Y = 0.3199 X + 38.797	0.9172	35.01 ± 0.024
TTO	Y = 0.8103 X + 27.977	0.9593	27.18 ± 0.028

Figure 1. In vitro anti-inflammatory activity of essential oil (KPO and TTO)

Figure 1 displays the concentration-dependent anti-inflammatory activity of essential oils against BSA denaturation. The reference drug, diclofenac sodium, exhibited potent anti-inflammatory activity, boasting the highest IC_{50} value of 17.67 ± 0.023 µg/mL. Meanwhile, TTO also demonstrated significant inhibition against protein denaturation with an IC50 value of 27.18 ± 0.028 µg/mL. This is in alignment with the value reported by John R.R et al. 2017 [32] which listed an IC50 value of 29.391 µg/mL. In contrast, KPO showed the least potential in preventing protein denaturation, with an IC50 value of 35.01 ± 0.024 µg/mL.

Physiochemical Evaluation of Emulgel

The emulgel was a shiny, jelly-white product that spread easily and had a firm, smooth texture. It possessed the specific aroma of eucalyptus and citrus peel (Figure 2). All emulgels produced a cooling sensation and adhered well to the skin, yet they could be easily removed with tap water. The pH of the prepared emulgel was slightly acidic (5.95 ± 0.055), which enhances permeability as it falls within the human skin pH range of 4.5-7.0 [33, 34]. All prepared emulgels exhibited good homogeneity upon visual and microscopic examination, and the average viscosity index was comparable to previous studies [35, 36]. Moreover, emulgels maintained their

homogeneity at different temperatures of 4, 25, and 40°C. Generally, all emulgels met the acceptable consistency conditions for application.

Figure 2. Emulgel state recorded at 25 oC

Stability Evaluation

The stability test confirmed that there were no statistically significant changes at room temperature (25oC) in pH ($p = 0.489$), viscosity ($p = 0.512$), and spreadability index ($p = 0.648$) (Table 2). The pH was observed to be under 6.0, which is considered acceptable to avoid the risk of irritation upon application to the skin. Thus, the formulation can be used in further effectiveness or clinical evaluations and scaled up for stability assessments in research and development scenarios.

Table 2. Stability of emulgel for a 2-month duration at 25°C

	Day 0	Day 15	Day 30	Day 45	Day 60
Organoleptic	Smooth, jelly white, well-homogenized, and greasy when applied.				
pH	5.95 ± 0.055	5.88 ± 0.087	5.83 ± 0.046	5.65 ± 0.562	5.95 ± 0.161
Viscosity (cPs)	4767.67 ± 88.94	4718.33 ± 22.74	4760.33 ± 40.67	4745.33 ± 22.37	4716.33 ± 42.64
Spreadability (kg.m/s·10^{-4})	10.83 ± 0.06	10.83 ± 0.23	11.33 ± 0.15	10.93 ± 0.06	10.80 ± 0.36

Each value represents the mean ± SD (n = 3); $p > 0.05$.

Acute Skin Irritation

Skin irritation is characterized by the formation of erythema and edema, with the level of irritation calculated based on the PII [31], as presented in Table 3. As no erythema or edema was recorded on the mice throughout the test, the calculated PII was 0, indicating no irritation. The hair on the mice's backs was observed to grow normally after 7 days of the test.

Table 3. Skin irritation testing of emulgel

Irritation Score	24 h	48 h	72 h	7 days
Erythema	No case	No case	No case	No case
Edema	No case	No case	No case	No case
PII	0.0	0.0	0.0	0.0

Mouse's skin after shaved

Mouse's skin after 1-week assay

Anti-inflammatory Activity of Emulgel on Carrageenan-induced Hind Paw Edema in Mice

The volume of the mice's hind paws was measured at 3 and 24 hours after carrageenan injection, and the level of inflammation reduction in all groups of mice was recorded, as summarized in Table 4. In general, there was a statistically significant difference in the inhibition percentage of protein denaturation among the tested groups after 3-hour treatment. However, there was no statistically significant difference within each of the six groups when comparing data obtained between the 3-hour and 24-hour treatments.

Table 4. Paw volume was documented in testing on carrageenan-induced hind paw edema in mice.

Group of mice (n = 8)	3 h		24 h	
	Pv (mL)	% Inhibition	Pv (mL)	% Inhibition
I	1.05 ± 0.05	0.00	0.92 ± 0.04	0.00
II	0.66 ± 0.05	37.14	0.59 ± 0.05	35.87
II	0.58 ± 0.05	44.76	0.49 ± 0.03	46.74
IV	0.84 ± 0.01	20.10	0.81 ± 0.05	11.97
V	0.76 ± 0.03	27.62	0.69 ± 0.03	24.97
VI	0.93 ± 0.06	11.43	0.88 ± 0.05	4.35

Each value represents the mean ± SD (n = 8); *$p < 0.05$ compared to control (I and VI)*

The emulgel loaded with a combination of essential oils significantly reduced the level of inflammation in a carrageenan-induced hind paw mouse model, compared to both the physiological control group and the negative control group. The combined essential oil emulgel demonstrated anti-inflammatory effects similar to those of diclofenac sodium. Conversely, the emulgel base alone was incapable of reducing inflammation compared to the physiological control. Both individual essential oils, TTO and KPO, displayed substantial inflammation reduction when compared to the physiological and negative control groups. However, samples that contained the combined essential oils demonstrated significantly higher anti-inflammatory activity in the carrageenan-induced hind paw edema mouse model compared to the samples loaded with a single essential oil. This effectively showcases the synergistic anti-inflammatory properties of the combined essential oil-loaded emulgel. After the third hour, the largest recorded percentage of inflammation inhibition was 44.76% yield by the group treated with the combined essential oils. This was followed in succession by the diclofenac-treated group (37.14%), TTO-treated group (27.62%), and KPO-treated group (20.10%). A similar trend was also observed after a 24-hour treatment period, with the anti-inflammatory activity of the combined essential oils-treated group recorded at 46.74%, followed by the diclofenac-treated group (35.87%), TTO-treated group (24.97%), and KPO-treated group (11.97%).

The data obtained from the current research demonstrated that the combination of TTO and KPO exhibited more pronounced anti-inflammatory effects on the carrageenan-induced hind paw edema mouse model than did a single essential oil. It is noteworthy that carrageenan-induced hind paw edema is a conventional in vivo model for acute inflammation [37]. This model is typically used to investigate new anti-inflammatory agents and drugs derived from natural sources. The inflammation induced by carrageenan is usually characterized by the release of inflammatory and pro-inflammatory mediators, including prostaglandins, cytokines, histamines, and bradykinins. Inflammation also involves the overproduction of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) and may activate certain enzymes like cyclooxygenase (COX). According to

previous studies, the anti-inflammatory efficacy of essential oils can be largely attributed to the main components present in both KPO and TTO, including terpinen-4-ol, γ-terpinene, α-terpinene, p-cymene, α-terpineol, limonene, eucalyptol, citronellal, β-pinene, and sabinene. These phyto-components have been extensively studied using modern techniques and their mechanisms of action have been partially elucidated, playing a key role in interaction with components resulting from inflammation induced by carrageenan. Our findings contribute to the scientific understanding of herbal medicine and highlight the considerable potential of the synergistic anti-inflammatory effectiveness of combined essential oils from KPO and TTO. This combination could be potentially utilized in developing plant-based therapeutic agents for the topical treatment of various skin conditions, particularly those related to the immune system, such as atopic dermatitis.

CONCLUSION

In conclusion, this research demonstrates the synergistic anti-inflammatory efficacy of combined KPO and TTO-incorporated emulgel in a carrageenan-induced hind paw edema mouse model. We hope these findings will encourage further studies to elucidate the mechanisms behind the potential synergistic interaction between the natural intrinsic components of KPO and TTO. The ultimate goal is to exploit the potential of these valuable essential oils for topical treatment against skin conditions.

Acknowledgment

The authors express their gratitude to the Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University-Ho Chi Minh City, for generously providing the necessary facilities for the study.

Conflicts of Interest

We declare that there is no conflict of interest.

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صياغة وتقييم جل مستحلب عشبي يحتوي على زيوت عطرية مجمعة من نبات *Melaleuca Alternifolia* و *Citrus hystrix*

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ملخص

الأهداف :تهدف هذه الدراسة إلى دراسة التأثيرات التآزرية المضادة للالتهابات للزيوت العطرية المركبة من *Melaleuca Alternifolia* وقشر الحمضيات.

المنهجية :تم تطبيق هلام ذو أساس مستحلب يحتوي على زيوت أساسية مجمعة من Melaleuca البديل وقشر الحمضيات موضعياً على المنطقة المحقونة من نموذج الفئران الوذمة الخلفية التي يسببها الكاراجينان .تم استخدام ديكلوفيناك الصوديوم كعنصر تحكم إيجابي .تم إخضاع جميع الفئران لتقييم نسبة تثبيط تمسخ البروتين والتي تم حسابها على أساس حجم المخلب .(Pv)

النتائج :تم تسجيل التأثيرات المضادة للالتهابات على وذمة المخلب الخلفي الناجمة عن الكاراجينان في الفئران في الساعة الثالثة مع أعلى نسبة تثبيط قدرها 44.76٪ في الفئران المعالجة بالزيوت العطرية مجتمعة تليها الفئران المعالجة بالديكلوفيناك 37.14)٪ (وشجرة الشاي .زيت أساسي معالج (27.62%)، وقشر الليمون الكافيري معالج بالزيت الأساسي .(20.10%) وبالمثل، لوحظ نفس الاتجاه أيضًا بعد العلاج لمدة 24ساعة مع النشاط المضاد للالتهابات للفئران المعالجة بالزيوت العطرية المجمعة المسجلة بنسبة 46.74%، يليها الفئران المعالجة بالديكلوفيناك (35.87%)، والفئران المعالجة بالزيوت العطرية لشجرة الشاي .(24.97%)وقشر الليمون الكافير المعالج بالزيت العطري .(11.97%)

الاستنتاج :أكدت هذه النتائج التأثيرات التآزرية المضادة للالتهابات للجيل القائم على مستحلب الزيوت العطرية المدمجة على نموذج الفئران الوذمة الخلفية التي يسببها الكاراجينان.

الكلمات الدالة: شجرة الشاي، الحمضيات، الزيوت العطرية، مضاد للالتهابات، الكاراجينان، الوذمة.

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تاريخ استلام البحث: 2023/8/1 وتاريخ قبوله للنشر 2023/12/21.